CLAIMS

1. Method for producing a plurality of pairs of sense and antisense composite primers, said plurality being specially adapted to the quantitative multiplex amplification of a plurality of target nucleotide sequences present in a nucleic acid or a mixture of nucleic acids, wherein each one of said sense or antisense composite primers produced consists:

- of a hybridization segment, respectively sense or antisense, which pairs with said nucleic acid or mixture of nucleic acids, so as to constitute a sense or antisense primer for one of the target nucleotide sequences of the plurality targeted, and
- of a nucleotide tag which is attached to the 5' end of said hybridization segment, but which does not pair with said nucleic acid or mixture of nucleic acids,
- and, optionally, of a non-nucleotide component,

characterized in that the respective sequences of the sense and antisense composite primers of said plurality of pairs are selected such that:

a) each sense composite primer has, within said plurality, an antisense composite primer with which it forms a pair of sense and antisense composite primers whose respective hybridization segments constitute, with respect to one another, a pair of sense and antisense primers for one of said target nucleotide sequences, each one of said target nucleotide sequences of the plurality targeted thus having a pair of sense and antisense composite primers which is intended for its amplification,

b) all the sense composite primers contain the same nucleotide tag and all the antisense composite primers contain the same nucleotide tag, the tag

of the sense composite primers being different from that of the antisense composite primers,

c) the sequence of the tag of the sense composite primers is absent from said nucleic acid or mixture of nucleic acids, or, at the very least, is only present therein at a frequency at least two times less than that predicted statistically for a random sequence of the same length, and the sequence of the tag of the antisense composite primers is absent from said nucleic acid or mixture of nucleic acids, or, at the very least, is only present therein at a frequency at least two times less than that predicted statistically for a random sequence of the same length,

d) the melting temperature of each composite primer (whether it is a sense or antisense primer) has a value 10 to 15°C higher (limits inclusive) than that which its hybridization segment would exhibit when naked without tag,

e) each composite primer of said plurality of pairs has a sequence such that no composite primer of said plurality of pairs can form, with itself or with another composite primer of the same plurality, complete or partial base pairing for which the variation in free energy ΔG associated with the formation of this possible pairing would be greater than 14 kcal/mol, said variation in free energy ΔG being calculated using the "*Primer Premier*" software version 5.0 marketed by PREMIER Biosoft International,

and in that the thus selected plurality of antisens composite primer pairs is synthesized.

2. Plurality of pairs of sense and antisense composite primers specially adapted to the quantitative multiplex amplification of a plurality of target nucleotide sequences present in a nucleic acid or a mixture of nucleic acids, characterized in that it is obtainable using the method according to claim 1.

3. Plurality of pairs of sense and antisense composite primers according to Claim 2, characterized in that said nucleic acid or mixture of nucleic acids is derived from mammalian cells.

4. Plurality of pairs of sense and antisense composite primers according to either one of Claims 2 and 3, characterized in that said nucleic acid or mixture of nucleic acids is derived from human cells.

5. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 4, characterized in that said nucleic acid or mixture of nucleic acids corresponds to the total genomic DNA of an organism or of a microorganism.

6. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 4, characterized in that said nucleic acid or mixture of nucleic acids corresponds to complementary DNA produced *in vitro* from RNA of an organism or of a microorganism.

7. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 6, characterized in that the tag of the sense composite primers, and also that of the antisense composite primers, each comprise, independently of one another, from 8 to 18 nucleotides, preferentially from 8 to 15 nucleotides, more preferentially 8 to 14 nucleotides, even more preferentially 9 to 12 nucleotides, very preferentially 10 nucleotides.

8. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 7, characterized in that the sequence of the tag of the sense composite primers, and also that of the tag of the antisense composite primers, each consist of a chain of 10 nucleotides the GC content of which is

between 20% and 60% (limits inclusive), preferentially between 20% and 50% (limits inclusive), very preferentially a GC content of 40%.

9. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 8, characterized in that the tag of the sense composite primers and/or that of the antisense composite primers is/are selected from the group consisting of the sequence of SEQ ID NO:1, the sequence of SEQ ID NO:2, and the sequences complementary thereto, SEQ ID NO:47 and SEQ ID NO:48.

10. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 9, characterized in that it comprises at least one pair of sense and antisense composite primers the respective sense and antisense hybridization segments of which have the following sequences:

- the sequences of SEQ ID NO:3 and SEQ ID NO:4, or
- the sequences of SEQ ID NO:7 and SEQ ID NO:8, or
- the sequences of SEQ ID NO:9 and SEQ ID NO:10, or
- the sequences of SEQ ID NO:11 and SEQ ID NO:12, or
- the sequences of SEQ ID NO:13 and SEQ ID NO:14.

11. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 9, characterized in that it comprises at least one pair of sense and antisense composite primers the respective sense and antisense hybridization segments of which have the following sequences:

- SEQ ID NO:27 and SEQ ID NO:28, or
- SEQ ID NO:29 and SEQ ID NO:30, or
- SEQ ID NO:31 and SEQ ID NO:32, or
- SEQ ID NO:33 and SEQ ID NO:34, or
- SEQ ID NO:35 and SEQ ID NO:36.

12. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 11, characterized in that the composite primers of said plurality of pairs each have a hybridization segment the melting temperature Tm of which is between 50 and 65°C, preferentially between 58 and 62°C, all limits inclusive.

13. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 12, characterized in that the composite primers of said plurality of pairs each have a melting temperature Tm of greater than 65°C, preferentially of between 68°C and 72°C, all limits inclusive.

14. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 13, characterized in that said non-nucleotide component is a label for detecting nucleotide products.

15. Plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 14, characterized in that it comprises from 2 to 15 pairs of sense and antisense composite primers.

16. Method for producing a plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 15, characterized in that it comprises the following steps:

a) selected from:

- pairs of sense and antisense hybridization segments which each form a pair of sense and antisense primers for one of said target nucleotide sequences, and
- nucleotide tags which are absent from said nucleic acid or mixture of nucleic acids, or which at the very least are only present therein at a frequency at least

two times less than that predicted statistically for a random sequence of the same length,

are a plurality of pairs of sense and antisense hybridization segments which covers the plurality of target nucleotide sequences targeted, and a pair of nucleotide tags,
the respective sequences of which are such that:

when one of the two selected tags is attached to the 5' end of each selected sense hybridization segment, and the other of the two selected tags is attached to the 5' end of each selected antisense hybridization segment, then:

- each resulting sense or antisense composite primer has a melting temperature Tm with a value 10 to 15°C greater (limits inclusive) than that which its hybridization segment would exhibit when naked without tag, and
- each resulting sense or antisense composite primer has a sequence such that it cannot form, with itself or with another resulting composite primer, a complete or partial base pairing for which the variation in free energy ΔG associated with the formation of this pairing would be greater than 14 kcal/mol,

b) the plurality of pairs of sense and antisense composite primers which results from the selection of the plurality of pairs of hybridization segments and of the pair of tags made in step a), and of the addition of the sequence of one of the two selected tags to the 5' end of each sense hybridization segment of the selected plurality, and of the addition of the sequence of the other of the two selected tags to the 5' end of each antisense hybridization segment of the selected plurality, is produced.

17. Method for producing a plurality of pairs of composite primers according to any one of Claims 2 to 15, characterized in that it comprises the following steps:

a) a plurality of pairs of sense and antisense hybridization segments is selected:

- in which each pair of segments constitutes a pair of sense and antisense primers for each one of the target nucleotide sequences targeted, and
- in which no segment can form, with itself or with another segment of this plurality, a complete or partial base pairing for which the variation in free energy ΔG associated with the formation of this possible pairing would be greater than 14 kcal/mol, preferentially 13 kcal/mol, more preferentially 12 kcal/mol,

b) two nucleotide tags are selected:

- the respective sequences of which are absent from said nucleic acid or mixture of nucleic acids, or at the very least which are only present therein at a frequency at least two times less than that predicted statistically for a random sequence of the same length, and
- which have respective sequences such that their addition, for one, to the 5′ end of each one of the sense hybridization segments selected in step a) and, for the other, to the 5′ end of each one of the antisense hybridization segments selected in step a), does not produce a set of sense and antisense composite primers within which a composite primer would be capable of forming, with itself or with another composite primer of this set, a complete or partial base pairing, the formation of which would correspond to a variation in free energy ΔG of greater than 14 kcal/mol,

c) a plurality of pairs of sense and antisense composite primers is produced by adding the sequence of one of the two tags selected in step b) to the 5′ end of each sense hybridization segment selected in step a), and by adding the sequence of the other of the two tags selected in step b) to the 5′ end of each antisense hybridization segment selected in step a), whereby a plurality of pairs of composite primers according to any one of Claims 2 to 15 is produced.

18. Method according to Claim 17, characterized in that said hybridization segments, whether they are sense or antisense, each have (in the absence of a tag) a melting temperature Tm of between 50 and 65°C (limits inclusive).

19. Plurality of pairs of composite primers according to any one of Claims 2 to 15, characterized in that it can be obtained using a method according to any one of Claims 16 to 18.

20. Pair of sense and antisense composite primers suitable for use within a plurality according to any one of Claims 2 to 15, characterized in that it is selected from a plurality according to Claim 19.

21. Method for producing a pair of tags suitable for use as a tag of sense composite primers and a tag of antisense composite primers in a plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 15 and 19, which pair is termed universal, characterized in that it comprises the following steps:

a) at least 30 pairs of sense and antisense hybridization segments are selected:

- which each form a pair of sense and antisense primers for a nucleotide target, so as to target at least 30 different nucleotide targets on said nucleic

acid or mixture of nucleic acids, and taking care that these at least 30 targets exhibit a uniform distribution throughout the length of said nucleic acid or mixture of nucleic acids, or at the very least in the region(s) in which are found the target nucleotide sequences whose amplification in multiplex is desired, and

- each segment of which has a melting temperature Tm of between 50 and 65°C (limits inclusive),

thus constituting a set of pairs of test sense and antisense segments,

b) for each pair of test segments of the set, the maximum value of the variation in free energy ΔG that this pair can exhibit, by partial or complete base pairing of each one of the two segments with itself or with the other segment of the same pair, is determined,

c) two tags of different sequences are selected:

- which are not present in said nucleic acid or mixture of nucleic acids, or at the very least which are only present therein at a frequency at least two times less than that predicted statistically for a random sequence of the same length, and
- the addition of which, for one, to the 5′ end of each test sense segment and, for the other, to the 5′ end of each test antisense segment, leads to an increase of a value of between 10 and 15°C (limits inclusive) in the melting temperature Tm of each one of the test segments, and
- the addition of which, for one, to the 5′ end of each test sense segment and, for the other, to the 5′ end of each test antisense segment, does not for any of the pairs of test sense and antisense segments lead to an increase of more than 3 kcal/mol in said maximum value ΔG determined for each test pair in step b),

d) the two selected tags are produced.

22. Pair of nucleotide tags intended to cooperate with a plurality of sense and antisense hybridization segments so as to form a plurality of pairs of sense or antisense composite primers according to any one of Claims 2 to 15 and 19, which pair is termed universal, characterized in that it is obtainable by the method according to Claim 21.

23. Pair of nucleotide tags according to Claim 22, characterized in that it is selected from the group of pairs of tags of respective sequences:

- the sequences SEQ ID NO:1 and SEQ ID NO:2,
- the sequence SEQ ID NO:1 and the sequence complementary to SEQ ID NO:2 (SEQ ID NO:48),
- the sequence complementary to SEQ ID NO:1 (SEQ ID NO:47) and the sequence SEQ ID NO:2,
- the sequence complementary to SEQ ID NO:1 (SEQ ID NO:47) and the sequence complementary to SEQ ID NO:2 (SEQ ID NO:48).

24. Nucleotide tag suitable for use as a tag in the sense composite primers or in the antisense composite primers of a plurality according to any one of Claims 2 to 15 and 19, characterized in that it is selected from a pair of tags according to Claim 22 or 23.

25. Nucleotide tag suitable for use as a tag in the sense composite primers or in the antisense composite primers of a plurality according to any one of Claims 2 to 15 and 19, characterized in that its sequence:

- consists of 10 nucleotides,
- has a GC content of between 20% and 60% (limits inclusive), preferentially between 20% and 50% (limits inclusive), very preferentially a GC content of 40%, and
- is absent from said nucleic acid or mixture of nucleic acids, or at the very most is only present therein at a frequency at least two times less than

that statistically predicted for a random sequence of the same length.

26. Nucleotide tag according to Claim 25, characterized in that, in addition, its sequence is such that the complete pairing with the chain of 10 nucleotides which constitutes the sequence completely complementary thereto exhibits a free energy of formation ΔG which does not exceed 11 kcal/mol.

27. Tag according to either one of Claims 25 and 26, characterized in that its sequence is selected from the group consisting of the sequence of SEQ ID NO:1, the sequence of SEQ ID NO:2, and the sequences complementary thereto, SEQ ID NO:47 and SEQ ID NO:48.

28. Sense or antisense composite primer suitable for use within a plurality according to any one of Claims 2 to 15, characterized in that it is selected from a pair of primers according to Claim 20, and in that the tag which it contains is a tag according to any one of Claims 24 to 27.

29. Method for amplifying in multiplex a plurality of target nucleotide sequences present in a nucleic acid or a mixture of nucleic acids, by hybridizations and elongations of a plurality of pairs of amplification primers, characterized in that said plurality of pairs of amplification primers is a plurality of pairs of sense and antisense composite primers according to any one of Claims 2 to 15 and 19.

30. Method of amplification according to Claim 29, characterized in that said plurality of target nucleotide sequences amplified in multiplex consists of 2 to 16 target sequences.

31. Method of amplification according to Claim 29 or 30, characterized in that said target nucleotide

sequences consist of 90 to 500 bp, preferentially of 90 to 300 bp.

32. Method for the quantitative multiplex amplification of a plurality of target nucleotide sequences present in a nucleic acid or a mixture of nucleic acids, by hybridizations and elongations of a plurality of pairs of amplification primers, characterized in that the method according to any one of Claims 29 to 31 is used, carrying out said hybridizations and/or elongations in the presence of an agent which facilitates DNA strand separation, such as dimethyl sulphoxide (DMSO) or triethylammonium acetate (TEAA).

33. Method of amplification according to Claim 32, characterized in that said hybridizations and elongations are carried out with successive hybridization-elongation-denaturation cycles until the amplifications of said target nucleotide sequences have exponential phase kinetics.

34. Method of amplification according to either one of Claims 32 and 33, characterized in that all the pairs of composite primers are used in equimolar concentration.

35. Method for determining the presence or absence of at least one genomic rearrangement within a genetic material B relative to a reference genetic material A, characterized in that:

- at least one nucleotide target which constitutes a marker for the rearrangement(s) sought is selected, and in that
- the method of amplification according to any one of Claims 29 to 31, or else that according to any one of Claims 32 to 34, is applied to said genetic material B, using, for each target selected, a pair of composite pri[redacted]s which is selected from [redacted]plurality of

pairs of composite primers according to any one of Claims 2 to 15 and 19, and which is suitable for the amplification of this target from the genetic material B,

said material B being considered as exhibiting said genetic rearrangement when the result of amplification of said at least one marker target, obtained from the material B, is significantly different from that which is obtained from the reference material A under identical conditions, and

said material B being considered as not exhibiting said genetic rearrangement when the result of amplification of said at least one marker target, obtained from the material B, is not significantly different from that which is obtained from the reference material A under identical conditions.

36. Method according to Claim 35, characterized in that said at least one genomic rearrangement is a gene rearrangement.

37. Method according to Claim 35, characterized in that said at least one genomic rearrangement is a chromosomal rearrangement.

38. Method according to any one of Claims 35 to 37, characterized in that said genetic material B comprises at least one human gene.

39. Method for determining at least one of the limits of one or more genomic rearrangement(s) which has (have) been detected within a genetic material B by comparison with a reference genetic material A, characterized in that:

a) a candidate region within which said at least one limit is potentially located is selected,

b) for each rearrangement, a set of nucleotide targets is chosen, among which at least one is chosen to constitute a marker for this rearrangement, the

other target(s) being chosen on both sides or on one or other sides of this marker target inside the candidate region chosen in step a) so as to cover the extent of this candidate region,

c) the method according to any one of Claims 29 to 31, or else the method according to any one of Claims 32 to 34, is applied to said genetic material B, using, for each target of said chosen set, at least one pair of composite primers which is chosen from a plurality of pairs of composite primers according to any one of Claims 2 to 15 and 19, and which is suitable for amplifying this target from said genetic material A,

d) for each target, the intensity of amplification thus obtained from said genetic material B is measured, and it is compared to the control intensity which is obtained for this same target under the same conditions but by applying said method of amplification to said reference genetic material A,

e) it is determined whether, within the chosen set of targets, at least one target is amplified with an intensity not significantly different from the control intensity, and, if this is not the case, steps a) to e) are repeated while broadening the candidate region chosen in step a),

said at least one limit of the or of each one of the rearrangements within said genetic material B being considered to be within a zone between:

- the position of the marker target for said rearrangement, and
- the position of the target which has been amplified with an intensity not significantly different from the control intensity or, if there are several of them, with that which is closest to said marker target,

f) if desired, the precision of determination of said limit is refined, by gradually walking into the zone determined in step e) above, by repeating steps a) to e), choosing as candidate region in step a) the zone identified in the immediately preceding step e), and

choosing in step b) a set of nucleotide targets which covers this zone identified in step e).

40. Method for producing a genomic rearrangement map, characterized in that the limits of at least one genomic rearrangement are determined using the method according to Claim 39, and in that these limits are recorded on a gene or chromosome map.

41. Method for identifying, and optionally isolating, at least one gene liable to be involved in a genetic disease, characterized in that:

- the method for determining the presence or absence of at least one genomic rearrangement according to any one of Claims 35 to 38 is carried out on a genetic material B derived from organisms exhibiting said genetic disease, a genomic material which is comparable but derived from control organisms serving as reference genomic material A, so as to detect the rearrangement(s) present in the material B relative to the material A, and in that

- the gene(s) affected by the detected rearrangement(s) is (are) identified, and optionally isolated,

this (these) identified and optionally isolated gene(s) corresponding to the gene(s) liable to be involved in said genetic disease.

42. Method for diagnosing a genetic disease from which an individual might suffer, or for estimating a propensity for this individual to develop such a disease, characterized in that the method for determining the presence or absence of at least one genomic rearrangement according to any one of Claims 35 to 38 is carried out on a sample of genetic material representative of said genetic disease, and in that said diagnosis is considered to be positive, or, where appropriate, said propensity is considered to be high, when said at least one genetic rearrangement is

determined as being present in said sample and, conversely,
said diagnosis is considered to be negative, or, where appropriate, said propensity is considered to be low, when said at least one genomic rearrangement is determined as being absent from said sample.

43. Kit for carrying out a method of amplification according to any one of Claims 29 to 31, and/or a method of amplification according to any one of Claims 32 to 34, and/or a method for determining the presence or absence of at least one genomic rearrangement according to any one of Claims 35 to 38, and/or a method for determining the limits of one or more genetic rearrangement(s) according to Claim 39, and/or a method for identifying at least one gene involved in a genetic disease according to Claim 41, and/or a diagnostic or prognostic method according to Claim 42, characterized in that it comprises at least one pair of composite primers according to Claim 20, and/or at least one composite primer according to Claim 28, and/or at least one tag according to any one of Claims 24 to 27, optionally combined with an amplification primer and/or with a label for detecting nucleotide products.